MULTI-DIMENSIONAL LIQUID CHROMATOGRAPHY SEPARATION SYSTEM

BACKGROUND OF THE INVENTION

[01] Liquid chromatography is a basic separation technique that has been well established for chemical, biological, biochemical, environmental, and other analyses.

[02] There are many principles of liquid chromatographic separation modes that have been known. Commonly, normal phase adsorption, reverse phase, ion exchange, or size exclusion modes are employed, but usually a single separation mode among these can be used successfully for liquid chromatographic analysis. If two or more separation modes could be combined orthogonally, a power of multiple modes of separation could be applied to a complex sample mixture.

[03] Generally speaking, one liquid chromatographic system has a single pathway or mechanism for mobile phase control. Thus, when two or more different kinds of solid phase columns (after this, "column") are used, they are limited to a single mobile phase, or one kind column is used with a multiple selection valve for mobile phases. Alternatively, the analytes separated and eluted from a 1st column are collected when they elute. Subsequently, these are re-injected into 2nd system combined with a 2nd column using a 2nd mobile phase in a batch-wise process. If the mobile phase from the first separation is incompatible with the second column, an intermediate step, such as desalting or concentration, is implemented.

[04] In the case of biological or clinical samples, the sample matrix is usually very complex.

[05] Batch or two-step sample-collection makes it difficult to implement an automated separation system, and adds the disadvantages such as the loss of the analytes during transfer and the inconvenience of batch processing.

[06] Using a combination between independent multiple systems based on orthogonal separation modes (such as ion exchange mode vs. reverse phase mode), it may be expected that the utilization of the different selectivity between target analytes and matrix contaminants will produce a much better separation. Because liquid chromatographs have only a single liquid flow path, it is necessary that multiple orthogonal systems be combined with columns and mobile phases integrated into one liquid chromatograph system.

[07] Liquid chromatograph systems that have at least two orthogonal systems combined with columns and mobile phases are disclosed in several cited papers as examples.

[08] For example, a first reference discloses a system in which analytes eluted from a 1st analytical column are trapped in two small volumes of sample tube on a switching valve (G.J. Opiteck *et al.*, Anal.Chem. 69 (1997) 1518-1524). These sample tubes are alternately interchanged, trapping from a fraction from the 1st column and depositing it onto a 2nd analytical column. In this technique, the dead volume of the sample tube for trapping causes deleterious effects for separation at the 2nd column. Furthermore, desalting cannot be performed because no trapping column is used.

[09] The second reference discloses a technique using a single trapping column for improved biological analysis (M.T. Davis *et al.*, J. Chromatogra. B 752 (2001) 281 – 291). In this reference, only three elution bands (such as flow through, starting load, bound on 1st column) were used. Thus, separation on the 1st column may not enough for most of the analytes if there were multiple fractions. Also each of three bands was trapped just before each of the 2nd dimension analysis. Even if more than three bands can be separated on 1st dimension side, delivery of 1st mobile phase needs to be stopped while 2nd analysis is performing in order to prevent from mis-eluting to the waste and losing the analytes tapped on the 1st column. Further desalting using different solvent from 1st mobile phase cannot be performed in this system configuration.

[10] Two similar techniques are disclosed in the third and fourth references (K. Wagner *et al.*, J. Chromatogr. A 893 (2000) 293 -305) and (G.J. Opiteck *et al.*, Anal.Biochem. 258 (1998) 349-361). In both of these references, the eluent from the 1st column flows onto the 2nd column directly. Both systems alternate between two parallel separate 2nd columns mounted onto column switching valves, and switch between trapping and separating. Because the 2nd columns are used for both trapping and for a 2nd dimension separation, the differences between column properties can be difficult to balance, negatively affecting the results, and decreasing reproducibility. Also, each 2nd column presents a high backpressure for 1st column. High backpressure may reduce the lifetime and performance of the 1st column.

[11] A fifth report discloses using 1st column and 2nd columns connected serially. Both 1st mobile phase and 2nd mobile phase are sent individually into both 1st and 2nd columns (A. J. Link *et al.*, Nat. Biotechnol. 17 (1999) 676-682). This system does not have independent paths for the 1st and 2nd systems.

[12] One common disadvantage among these reports is that desalting could not be performed before loading the analytes into a 2nd column when the effluent from 1st column requires salt containing buffers. Many choices for a second analytical chromatographic mode are incompatible with salt buffers for optimal separation. Additionally, because mass spectrometry is frequently used as a detector to provide sensitivity and selectivity, the samples (or solutions) containing non-volatile salts are incompatible with optimal performance. Deposition of salt interferes with electrospray ionization and transfer of the vaporized ions into the mass spectrometer.

[13] References are also given for the equipments, parts and techniques, which this invention utilizes:

The catalog of 14 port rotary valve (Valco Instruments Co. Inc., TX)

The catalog of LC-VP series (Shimadzu Corporation, Japan)

The catalog of CapTrap as used trapping column (Michrom BioResources, Inc., CA)

SUMMARY OF THE INVENTION

[14] In view of the problem described above, the object of the present invention is to provide a multi-dimensional liquid chromatograph separation system that can perform automatic separations of samples containing complex mixtures.

[15] A liquid chromatograph separation system according to the present invention that has properties includes at least two or more individual systems. Each of the systems has a mobile phase and a column and controls independently the mobile phase that flows through the column. The system has a plurality of trapping columns for trapping analytes with the mobile phase that are eluted from the column. In addition, the system has a mechanism for selecting either loading the analytes eluted from the column onto the trapping columns, or diverting the mobile phase to waste, and a mechanism for eluting the analytes trapped on each trapping column and for online loading onto a second analytical column.

[16] In another aspect of the present invention, the liquid chromatograph system further comprises a system for detection of separated analytes eluted from the second column or a last column if there is a series of more than two systems with more than two columns.

[17] In further aspect of the present invention, the liquid chromatograph system further comprises a system for detection of separated analytes eluted from the column or an intermediate column if there are more than two independent systems and columns.

[18] In still further aspect of the present invention, the liquid chromatograph system further comprises a system for desalting that is set up independently from any other systems. The desalting is performed after trapping the analytes on each trapping column and before loading onto the next column, and a solvent for desalting is different from those of any other mobile phase and mobile phase.

[19] Finally, all of these processes including injection and desalting process are performed continuously online without attendant, and uninterrupted. Many samples can be analyzed routinely and successively using this system. This provides an economic advantage by increasing through-put for complex mixture analyses using automation.

BRIEF DESCRIPTION OF THE DRAWINGS

[20] **Figure 1** is a schematic diagram of a multi-dimensional chromatograph separation system according to the first embodiment of the present invention.

[21] **Figure 2** is a schematic diagram of a rotary valve and trapping columns of **Figure 1**.

[22] **Figure3** is a schematic diagram of the multi-dimensional chromatograph separation system according to the second embodiment of the present invention using a valve combination instead of the rotary valve.

[23] **Figure 4** is a schematic diagram of the multi-dimensional chromatograph separation system according to the third embodiment of the present invention using another valve combination instead of the rotary valve.

[24] **Figure 5** is a schematic diagram of the multi-dimensional chromatograph separation system according to the fourth embodiment of the present invention using another desalting system.

[25] **Figure 6** is a time chart illustrating a sequence timeline of events using the present invention.

[26] **Figure 7** is an example of a chromatogram resulting from the use of the present invention.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[27] **Figures 1** and **2** show the first embodiment of the present invention. **Figure 6** shows a time chart of the operation of the analysis process of this embodiment. As illustrated in **Figures 1** and **2**, the multi-dimensional chromatograph separation system according to this embodiment comprises a 1st analysis system **26** having a 1st column **6** and 1st mobile phase, a 2nd analysis system **27** having a 2nd column **24** and 2nd mobile phase; and a trapping system **28** having switching valves **12, 13**, a rotary valve **14** and multiple trapping columns **15-20**. In addition, for desalting, this present invention incorporates a desalting solvent delivery system **29**.

[28] The mechanism and function of each part or component are described in detail in the following paragraphs:

[29] In the 1st analysis system **26**, 1st mobile phase A as **1-A** and B as **1-B** are loaded into their delivery pumps **3a, 3b** individually from each mobile phase reservoir **1a, 1b** through each degasser **2a, 2b**. Generally, a binary (or more) gradient elution technique is used in the main target area of this present invention; thus, this system also has a binary gradient elution system. Mobile phase A as **1-A** and B as **1-B** are mixed at a gradient mixer **4** and delivered into the 1st column **6** through an injector **5**. Either an automatic sampler that can inject a sample automatically or manual injector can be used as the injector **5**.

[30] The effluent containing target analytes eluted from the column **6** is loaded into a switching valve **12** of the trapping system **28** through a 1st detector **7**, if needed. Optionally, a non-destruction detector, such as a UV-Visible detector, can be used as the detector **7**.

[31] The 2nd mobile phase A as **2-A** and B as **2-B** of the 2nd analysis system are also loaded into their delivery pumps **10a**, **10b** individually from each mobile phase reservoir **8a**, **8b** through each degasser **9a**, **9b**. Both A as **2-A** and B as **2-B** of the 2nd mobile phase are mixed at a gradient mixer **11** in the same way as the 1st mobile phase. Then the 2nd mobile phase is delivered into the 2nd column **24** through the valve **12**.

[32] The effluent containing target analytes is loaded from the column **24** into a 2nd detector **25**. An electrospray ionization mass spectrometer is usually chosen as the detector **25** because of its high sensitivity and selectivity, and to structurally characterize eluting analytes.

[33] The trapping system **28** includes valves **12**, **13**, **14** and trapping columns **15**, **16**, **17**, **18**,**19**, **20**. One of the ports for trapping, desalting or elution to the 2nd column **24** is selected as switching shown on the timeline chart in **Figure 6**. Timeline **53** indicates the switching valve **13**, and timeline **54** indicates the switching valve **12**. Rotating the rotary valve **14** is performed stepwise at periods **41** to **52**.

[34] Each port on the switching valve **12**, **13** is connected as dotted line shown in **Figure 1** and timeline **53**, **54** in **Figure 6**, while the ports on the rotary valve **14** are set at **R1** and **R1'** at the time just after the sample is applied at injector **5**. This means that the effluent from the column **6** is loaded into ports **A3** and **A4** (through dotted line) on the switching valve **13**, next loaded into the trapping column **15** through the ports **R1** (before the trapping column **15**) and **R1'** (after the trapping column **15**) on the rotary valve **14** and then lorded to **Waste 1** port from ports **B2**, **B1** on the switching valve **12**. This process is performed during the time period **41** in the time chart of **Figure 6**.

[35] Incrementally, as shown in **Figure 2**, a pair of the ports of the rotary valve **14** is advanced to a pair such as **R2**, **R2'** and **R3**, **R3'**.... as the same period of step-wise gradient ratio (a concentration of the 1st mobile phase B solvent) increases to the next step. That is, for example, a pair of ports **R1**, **R1'** on the rotary valve **14** is changed into a pair of ports **R2**, **R2'** at the next period **42**. The analytes eluted from the column **6** will be trapped on the trapping column **16** in the same period. The retention time is different between analytes trapped on the column **15** and the analytes trapped on the column **16**. In other words, the properties of the analytes on the column **15**

(the former) differ from the analytes on the column **16** (the latter) because of the separation properties exhibited by the column **6**.

[36] Following this, each port is incremented, one-by-one, as well as increasing the step-wise gradient ratio. Analytes which have different retention times in the column 6 elute and are then trapped by the trapping columns **17, 18, 19, 20** in sequence. In this process, most of the effluent eluted from the column **6** is trapped on the columns **15** to **20** as if they were multiple fraction collectors.

[37] During the same period (period **41** to **46** in **Figure 2**), the 2nd mobile phase is loaded into the column **24** through ports **B3, B4** (via dotted line) on the switching valve **12**. Continuing to load the 2nd mobile phase into the column **24** serves to maintain the equilibrium state in the column **24**. During the same period, the desalting solvent is loaded from a reservoir **21** into a delivery pump **23** through a degasser **22**, and, further, is sent to ports **B6, B5** (via dotted line) on the switching valve **12**, after this, is sent to **Waste 2** port through **A5, A6, A2, A1** on the switching valve **13**.

[38] After all the trapping processes are finished, the position of the valve **13** is advanced as illustrated by a double line in the timeline **53** in **Figure 6**. The pair of the ports of the rotary valve **14** is changed back to the pair of the ports **R1, R1'** at the same time of the beginning of the period **47**. The effluent from the column **6**, which contains no material of analytical interest, is directed to waste from **Waste 2** through ports **A3, A2, A6** and **A1** (via double lines) on the valve **13** during this period. Meanwhile, the desalting solvent as **3-A** in Figure 1, during the desalt period **56** in **Figure 6**, is loaded into the column **15** through ports **B6, B5** (via dotted line) on the valve **12** and ports **A5, A4** (via double line) on the valve **13**, and **R1** on the rotary valve **14**, and after completion, it is flushed from **Waste 1** port through **R1'** on rotary valve **14** and ports **B2, B1** (via dotted line) on the valve **12**. Only salts are eluted from the trapping column **15** with the desalting solvent and washed out of the system. This desalting process on the trapping column **15** is performed at the period **56** on the timeline.

[39] The position of valve **12** is advanced as shown as a double line in the timeline scheme **54** after the desalting process. The 2nd mobile phase is loaded into the column **24** from **B3, B2** port on valve **12, R1'** on rotary valve, trapping column **15**, **R1** on rotary valve, **A4, A5** on valve **13** and **B5, B4** on valve **12**.

[40] The flow through the trapping column **15** is reversed relative to the trapping period; thus, the analytes trapped on the column **15** are back flushed onto the 2nd column **24**. The separation of the analytes within the column **24** is performed in the period **57** and the gradient program for the 2nd mobile phase is also run in the same period **57**. The desalting solvent is flushed from **Waste 1** port through **B6**, **B1** on the valve **12** during this period.

[41] At the beginning of period **48**, the port of rotary valve **14** is advanced to **R2**, **R2'**. Then for trapping the column **16**, the desalting process is performed in the period **58** and then back flushed into the column **24**. Separations in the column **24** are implemented in the period **59** as well as for the trapping column **15**.

[42] The same process is performed for trapping the column **17** in the period **49**, the column **18** in the period **50**, the column **19** in the period **51**, the column **20** in the period **52**, respectively; thus, all of the chromatograms for analytes trapped each trapping column are obtained individually.

[43] This present invention, using multiple trapping columns, enables efficient trapping of almost all analytes eluted from the column **6** as if there were multiple fractions collected and then loaded onto the column **24** as an automatic online process.

[44] Furthermore, the desalting process enables the liquid chromatograph to use the mass spectrometer as a detector **25** continuously and without the deleterious deposition of salts. Because the deliveries of both 1st and 2nd mobile phases are performed continuously, equilibrium conditions are maintained in the both 1st and 2nd columns at all times. The results of this mode of operation are better precision of analyses and preservation of column lifetimes.

[45] Because the column **24** is a single column used consistently for the 2nd dimension, this system does not suffer from retention or performance differences between two parallel columns like those used in the references **3** and **4** as mentioned above. The same level of reproducibility can be expected as the usual liquid chromatograph system. Even if each trapping column has a different property, the length and volume of the trapping column is much shorter and smaller than the analytical column, so the effect of the difference between trapping columns has little net effect. Also, the backpressure from each trapping column is lower than if a 1st analytical column were in series with a 2nd analytical column.

[46] Finally and most importantly, all of these processes are performed continuously online; thus, these processes are automatic, without attendant, and uninterrupted. This provides an economic advantage by increasing through-put for complex mixture analyses using automation.

[47] **Figures 3** and **4** show the second and third embodiments of the present invention. Either a combination of two 6-port 2-position switching valves **30** and **31** shown in **Figure 3** or a combination of a 6-port 2-position switching valve **32** and a 7-port manifold **33** shown in **Figure 4** can be used instead of a rotary valve for the same purpose.

[48] **Figure 3** shows the combination of two 6-port 2-position switching valves **30** and **31**, which can be used instead of the rotary valve **14**. Each port on 6-port 2-position switching valve **30** such as **P1, P2**, to **P6** is corresponded to **R1, R2** to **R6** on the rotary valve **14** shown in **Figure 1** and **2**. Each port on 6-port 2-position switching valve **31** such as **P1', P2'** to **P6'** is corresponded to **R1', R2'** to **R6'** on the rotary valve **14** in the same way. Combination of **P1** and **P1'** on each 6-port 2-position switching valve **30, 31** instead of **R1** and **R1'** on rotary valve **14** can be used in order to perform the same function as the first embodiment of the present invention, and another combination of ports are in the same way. When this second embodiment is used, each letter of **R1** to **R6** and **R1'** to **R6'** in each paragraph of this documents would be regarded as **P1** to **P6** and **P1'** to **P6'**.

[49] **Figure 4** shows the combination of a 6-port 2-position switching valve **32** and a 7-port manifold **33**, which can be used instead of the rotary valve **14**. Each port on 6-port 2-position switching valve **32** such as **Q1, Q2** to **Q6** is corresponded to **R1, R2** to **R6** on the rotary valve **14** shown in **Figure 1** and **2**. Each port on 7-port manifold **33** such as **Q1', Q2'** to **Q6'** is corresponded to **R1', R2'** to **R6'** on the rotary valve **14** in the same way. Combination of **Q1** and **Q1'** on each 6-port 2-position switching valve **32** and 7-port manifold **31** instead of **R1** and **R1'** on rotary valve **14** can be used in order to perform the same function as the first embodiment of the present invention. However, in this combination, all that have to be controlled is the 6-port 2-position switching valve **32** because all ports of **Q1', Q2'**, to **Q6'** on the manifold are always connected to the center common port as the 7^{th} port on the manifold **33**. When this third embodiment is used, each letter of **R1** to **R6** and **Q1'** to **Q6** in the paragraphs of this documents would be regarded as **Q1** to **Q6** and **Q1'** to **Q6'**.

[50] **Figure 5** shows the fourth embodiment of the present invention. For desalting or another solvent changing function, a binary solvent delivery system can be used as a 3rd solvent delivery system shown as **3-A, 3-B** in place of single desalting solvent delivery system shown in **Figure 5**. 3rd solvent may be different solvent from the 2nd mobile phase. For a 3rd solvent, an additional delivery system, can be used, consisting of solvent reservoir **34a, 34b** degasser **35a, 35b** delivery pumps **36a, 36b,** and a mixer **37** with a mobile phase selection valve **38**.

Example

[51] In order to clearly define the invention, the following example of its use is provided.

[52] The following example of a biochemical analysis is a separation of a mixture of enzyme-digested proteins. The example is a tryptic digest of proteins, including beta-casein, myoglobin, and bovine serum albumin, as typical proteins. It is known that many peptide fragments result from proteolytic digestion with trypsin. Consequently each peak in a single dimensional chromatographic analysis of this mixture will contain multiple components, making it difficult to identify each component in the mixture. Thus, this is a suitable example for the demonstration of this invention.

[53] The system is made up of definite sub-parts as follows:

[54] The 1st analysis system **26** contains each of the following components.

[55] The 1st mobile phase A as **1-A** was filled in the solvent reservoir **1a**, and 1st mobile phase B as **1-B** was filled in the solvent reservoir **1b**. In order to eliminate air dissolved in the mobile phase, degassers **2a, 2b** (e.g., DGU-14A; Shimadzu Corporation, Japan) were used. The 1st mobile phase as both of A as **1-A** and B as **1-B** were delivered using the delivery pumps **3a, 3b** (e.g., LC-10ADvp; Shimadzu Corporation, Japan) and were loaded to the autosampler used as the injector **5** (e.g., SIL-10ADvp; Shimadzu Corporation, Japan) through the fixed volume gradient mixer **4** (e.g., Gradient mixer; Shimadzu Corporation, Japan). A UV-Visible detector (e.g., SPD-10A(V)*VP*; Shimadzu Corporation, Japan), which is a non-destruction detector, can be used optionally as the 1st detector **7** when the analyst chooses to monitor the effluent elute from the column **6**.

[56] Almost all of the peptide analytes that can be eluted from column **6** are trapped on one of the six trapping columns **15** to **20** (e.g., Peptide CapTrap; Michrom BioResources, Inc., CA) in this system. Because of this efficient trapping, the monitoring of effluent using the detector **7** is not necessary in order to detect the analytes just after the 1st column **6**. (In fact, monitoring using the detector **7** was only used during the initial set-up and testing of this invention.)

[57] The 2nd analysis system **29** contains each of the following components.

[58] The 2nd mobile phase A as **2-A** was filled in the solvent reservoir **8a**, and the 2nd mobile as phase B as **2-B** was filled in the solvent reservoir **8b**. In order to eliminate air dissolved in the mobile phase, degassers **9a**, **9b** (e.g., DGU-14A; Shimadzu Corporation, Japan) were used in the same fashion of 1st analysis system. The 2nd mobile phase as both of A and B were delivered using the delivery pumps **10a**, **10b** (e.g., LC-10ADvp; Shimadzu Corporation, Japan) and were loaded to the fixed volume gradient mixer **11** (e.g., Gradient mixer; Shimadzu Corporation, Japan)

[59] The trapping system **27** consists of each following component.

[60] In addition to the six trapping columns **15** to **20** as mentioned above, two 6-port 2 position switching valves (e.g., FCV-12AH; Shimadzu Corporation, Japan) are used as valves **12**, **13** and a **14** port rotary valve (e.g., ST 6 position valve; Valco Instruments Co. Inc., TX) is used as the rotary valve 14.

[61] Further, an electrospray ion trap mass spectrometer (e.g., LCQ; Thermo Finnigan, CA) is used as the 2nd detector **25**.

[62] The desalting solvent delivery system contains each of the following components.

[63] Desalting solvent was filled in solvent reservoir **21**, and delivered by delivery pump **23** (e.g., LC-10ADvp; Shimadzu Corporation, Japan) through a degasser **23** (e.g., DGU-14A; Shimadzu Corporation, Japan) into the valve **12**.

[64] The mobile phase, columns, and chromatographic condition, which were used in this example, are as follows:

[65] [Chromatographic condition for 1st dimension analysis]

1st column **6** :

PolyLC PolySULFOETHYL A 50x1mm, 5 μm, 200Å)

1st Mobile Phase :

Solvent A as **1-A** in **1a** ; 10mM Formic acid / Ammonium formate buffer pH 4.0

Solvent B as **1-B** in **1b** ; Solvent A containing 100mM Ammonium sulfate

Step gradient program :

Solvent B 1% 10% 20% 30% 50% 99% ; each 5 min

Flow rate :

80μL/min

Temperature :

40 °C

[66] [Chromatographic condition for 2nd dimension analysis]

2nd column **24** :

Keystone BetaBasic C-18 0.3mm x 100mm, 5μm, 150Å)

2nd Mobile Phase :

Solvent A as **2-A** in **8a** ; Water / Acetonitrile / Formic acid = 95 / 5 / 0.1 (v/v)

Solvent B as **2-B** in **8b** ; Water / Acetonitrile / Formic acid = 20 / 80 / 0.1 (v/v)

Gradient program :

Solvent B 10% - 60% (start - 30min)

60% - 80% (30min - 35min), 80% (35min - 40min)

Flow rate :

10μL/min

Temperature :

40 °C

[67] [Trap columns / Desal]

Trap column :

Michrom BioResources, Inc. Peptide CapTrap 0.5mm x 2mm, 0.5μL

(15, 16, 17, 18, 19, 20)

Desalt solvent **21**:

Water / Formic acid = 100 / 0.1 (v/v) 80μL/min, 4.5min

[68] A detailed explanation of this example follows, step by step.

Step1

[69] The sample solution was applied using the injector **5** and loaded into the column **6**. Just after sample injection at the injector **5**, the position shown as a dotted line was selected as position of each valve **12, 13**, and the ports **R1, R1'** were selected as the position of the rotary valve **14**. That is, the effluent from the column **6** was loaded to the trapping column **15** through ports **A3, A4** on the valve **13** and **R1** on the rotary valve **14**, then was sent to **Waste 1** port from the column **15** through **R1'** on the rotary valve **14** and **B2, B1** on the valve **12**. During this period, the gradient concentration of the 1st mobile phase (concentration of B) was 1%, that is, the concentration of ammonium sulfate was 1mM. Accordingly, the analytes, which had been eluted from the column **6** by 1mM ammonium sulfate in the first **5** minutes period, were trapped on the trapping column **15**.

Step2

[70] Second, when the gradient concentration of the 1st mobile phase (concentration of B) became 10%, that is, the concentration of ammonium sulfate was 10mM, the position of the rotary valve **14** was changed into **R2, R2'**. In this period, the analytes, which had been eluted from column **6** by 10mM ammonium sulfate in the second **5** minutes period (show as **42** in time chart, **Figure 6**), were trapped on the trapping column **16**.

[71] In other words, the analytes trapped on the column **15** exhibited different ion exchange retention properties on column **6** relative to those analytes trapped on the column **16**.

Step3

[72] In the same way, the gradient concentration of 1st mobile phase (concentration of B) was increased, from 20% (20mM ammonium sulfate), 30% (30mM ammonium sulfate), and 50% (50mM ammonium sulfate) to 99% (99mM ammonium sulfate) in 5 min increments. During this time, the position of the rotary valve was changed into **R3-R3', R4-R4', R5-R5', R6-R6'** in succession shown as **43** to **46** in the timeline chart.

[73] As a result, each analyte that had different ion exchange properties in the column **6** was trapped sequentially in the columns **17, 18, 19, 20**. This process enables the

step-wise trapping of analytes eluted from the column **6** as if they were multiple fractions.

[74] The 2nd mobile phase was loaded into the column **24** through **B3**, **B4** on the valve **12** during the trapping period. Continuously flowing the mobile phase into the columns without a break retains an equilibrium state in the analysis system.

[75] The desalting solvent was loaded from the reservoir **21** into the delivery pump **23** through the degasser **22**. After this, the desalting solvent was sent to Waste **2** port through **B6**, **B5** on the valve **12** and **A5**, **A6**, **A2**, **A1** on the valve **13**.

Step4

[76] When the all trapping processes were finished, the position of the valve **13** was rotated into the position shown as double line. The port of the rotary valve **14** was changed back to **R1**, **R1'** at the same time at the beginning of the timeline period **47**. The effluent from the column **6**, which contained only residual analytes at this point, was flushed from **Waste 2** port through ports **A3**, **A2** and **A6** on the valve **13** during this period. The desalting solvent was loaded into the column **15** through **B6**, **B5** on the valve 12 and **A5**, **A4** on the valve **13** and **R1** on the rotary valve **14**, then eliminated from **Waste 1** port through **R1'** on the rotary valve **14** and ports **B2**, **B1** on the valve **12**. In the timeline period **56** (4.5 minutes), only the salt was eluted from the column **15** and was washed out of the system.

[77] In this desalting process, the analytes trapped on the column **15** were retained because the analytes had been trapped based on the hydrophobic interaction. In principle, few analytes are eluted by an aqueous desalting solution that does not contain organic solvents.

Step5

[78] The position of the valve **12** was changed as shown as a double line in the timeline scheme **54** after the desalting process. The 2nd mobile phase was loaded into the column **24** from ports **B3**, **B2** on the valve **12**, **R1'** on the rotary valve **14**, the trapping column **15**, **R1** on the rotary valve**14**, **A4**, A5 on the valve **12** and **B5**, B4 on the valve **13**.

[79] The flow through the trapping column **15** was reversed; thus, the analytes trapped on the column **15** were back flushed onto the 2nd column 24.

[80] The 2nd mobile phase has the solvent strength to elute the analytes from the trapping column **15** and load them onto the column **24**. The separation of analytes was performed on the column **24** in the timeline period **57**. The gradient program for the 2nd mobile phase had been programmed shown as axis **40** in **Figure 6**. The separation based on the hydrophobic interaction (reverse phase mode) at the column **24** was performed using this gradient elution program. The desalting solvent was at the same time eliminated through **Waste 2** port through **B6**, **B1** on the valve **12**.

[81] The analytes that had been separated on the column **24** were electrospray ionized into an ion trap mass spectrometer **25**. The output data obtained from the detector **25** was based on the relation between detection intensity and retention time and this output data can be plotted as a chromatogram of reconstructed ion intensities.

[82] The mass spectrometric reconstructed ion chromatogram for analytes trapped on the column **15** is shown as **68** in **Figure 7**. Each peak on this chromatogram corresponds to analytes of different abundance.

Step6

[83] In the same way, the same process was performed in timeline periods **48**, **49**, **50**, **51** and **52**. To affect these transitions, the position of the valve **14** was rotated to **R2**, **R2'** and the analysis of the analytes trapped on the trapping column **16** (eluted from the column **6** by 10mM ammonium sulfate) with desalting in the same way as **step4**. As the result, the chromatogram shown as **69** in **Figure 7** was obtained. Next, the chromatogram shown as **70** was obtained from the analytes trapped on the tapping column **17** (eluted from the column **6** by 20mM ammonium sulfate), the chromatogram shown as **71** from the ones on the column **18** (eluted from the column **6** by 30mM ammonium sulfate), the chromatogram shown as **72** from on the column **19** (eluted from column **6** by 50mM ammonium sulfate), the chromatogram shown as **73** from on the column **20** (eluted from column **6** by 99mM ammonium sulfate).

[84] According to these chromatograms, the analytes, which exhibit similar retention properties on the 2nd chromatographic analysis, were trapped exhibiting very different ion exchange properties. In other words, these analytes would not be separated by only a reverse phase analytical separation, but now can be distinctly characterized as a result of the orthogonal separation processes.

Appendix

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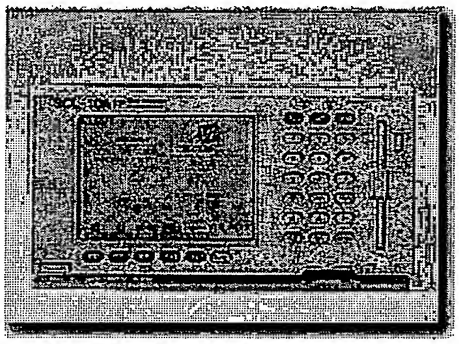

SCL-10AVP

System Controller for Single Point System Control

Shimadzu's new SCL-10A*VP* System Controller directly controls all VP Series components from it's new, simplified display menu for easy, user-friendly operation. All Shimadzu HPLC system modules are conveniently linked to the controller by fiber optic cables for swift "Plug-and-Play" set-up. Fiber optic interfacing enables low noise, high-speed data transmission which enhances system reliability and sensitivity. An optional SCSI interface allows multiple VP Series HPLC systems to be linked. Pair the SCL-10A*VP* with Shimadzu's powerful CLASS-VP™ chromatography software for unmatched control capability.

New User-Friendly Interface • Large, East-To-Read Characters and Icons • Customizable Display Menu and Start-Up Screen. • On-Screen "Help" • Graphical User Interface	The new controller features many improvements including a larger, more legible display and customizable menus which simplify system operation for novice and advanced users. Menus can now be tailored to see only the parameters that you care about. Function keys, detailed help information (with valid parameter ranges), and a graphical user interface streamline parameter setup and instrument operation.
GLP/GMP	Built-in validation support

Compliance	functions help you comply with GLP/GMP regulations and enhance productivity by reducing the labor required to perform validation tasks. A record of each module's operation is preserved and can be reviewed on-screen.
Transfer and Store Methods	Save method parameters to the built-in 3.5" disk drive for reference and for secure transfer to other VP Series HPLC systems. ROM version updates are downloaded from disk for easy upgradeability.
Simple Mode for Routine Analysis	Operate your isocratic system with a simplified parameter setup. Enter pump flow rate, detector wavelength, column oven temperature and autoinjector batch schedule parameters, and begin your run.
Advanced Mode for Full Control	The SCL-10A*VP*'s advanced mode accesses full VP Series capability, including the autoinjector's automated advanced sample pretreatment functionality. All operational parameters are controlled by the SCL-10A*VP*.
CLASS-VP™ Workstation Interface	Digital signals from VP Series detectors are rapidly transmitted from the SCL-10A*VP* to the CLASS-VP™ Chromatography Workstation by standard RS-232C interface. Analog signals from other manufacturers' detectors can be converted to digital by the SCL-10A*VP* (option). Recently acquired data is saved in the SCL-10A*VP* buffer, protecting against accidental PC failure.

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SHIMADZU North America

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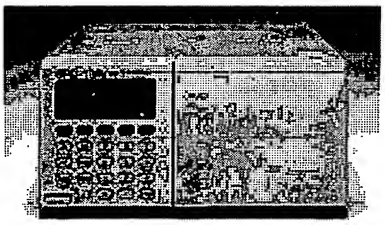

SPD-10A/10AVVP

High Sensitivity UV-Vis Detectors

The new SPD-10A*VP* and SPD-10AV*VP* VP Series UV and UV-Vis detectors combine ease of use and high sensitivity. Their optical design provides exceptionally low baseline noise, for unmatched performance. The SPD-10A*VP* and SPD-10AV*VP* deliver superb wavelength reproducibility, for high chromatographic stability. Wavelength programming optimizes component detection, and wavelength scanning allows absorbance spectra and lambda max determination. Maintenance is facilitated with front access to the flow cell and prealigned D_2 lamp which delivers double the lifetime (~2,000 hours). Simultaneous dual wavelength detection provides peak purity via ratio chromatogram for qualitative information from a single analytical run.

Unmatched Noise Specification ($\pm$0.35 x 10^{-5} AU)	Shimadzu's proven optical system delivers high sensitivity detection. The SPD-10A*VP* and SPD-10AV*VP* can detect low concentration peaks as well as peaks previously hidden in baseline noise - peaks that other detectors miss.
Baseline Stability for Reliable Analytical Results	Isolation of the light source compartment and of the monochromator reduces temperature variation, minimizing drift and reducing baseline noise.
Excellent Wavelength Reproducibility Ensures High Reliability	The high-precision monochromator grating drive mechanism has a high-resolution step motor and backlash-free force transmission system to give

	transmission system to give exceptional wavelength reproducibility (±0.1 nm). Stability is guaranteed with a wavelength accuracy specification of ±1 nm.
GLP/GMP Compliance	Built-in validation support functions help you comply with GLP/GMP regulations and enhance productivity by reducing the labor required to perform validation tasks. A record of each module's operation is preserved and can be reviewed on-screen.
Broad Wavelength Measurement Range	The SPD-10A*VP* uses a deuterium lamp for analysis in the wavelength range of 190 - 600 nm. The SPD-10AV*VP* measures in the 190 - 900 nm range and is equipped with both deuterium and tungsten lamps for maximum sensitivity in the visible region.
Simultaneous Dual-Wavelength Measurement	The SPD-10A*VP* and SPD-10AV*VP* can acquire data at two different wavelengths in a single analytical run. The ratio chromatogram output signal delivers qualitative information on peak purity, improving analysis reliability.
Wavelength Programming Exhibits Power in Multi-Component Analysis	Using the time program function, the optimum detection wavelength for each component peak can be used automatically in one run. Analysis of multi-components with different maximum wavelengths can be performed with higher sensitivity.
Qualitative Assurance by Wavelength Scanning	The wavelength scanning function creates an absorbance spectrum of the target component using the "stopped flow" technique. Since the background spectrum of the mobile phase can be subtracted, true spectrum are

	subtracted, true spectrum are attained.
Long-Life Deuterium Lamp	The new deuterium lamp for the SPD-10A*VP* and SPD-10AV*VP* lasts twice as long as before. Program the lamps to turn on and off automatically, further increasing useful lamp operation time.
Pre-Aligned Lamp Provides Easy Installation	The design of the SPD-10A*VP* and SPD-10AV*VP* enables fast, user-friendly lamp installation without time-consuming adjustments. The enhanced life of the deuterium lamp and new, pre-aligned lamp assembly design reduce detector maintenance.
Wide Array of Flow Cell Options	Optional flow cells for the SPD-10A*VP* and SPD-10AV*VP* support both micro and preparative applications. The cells of the SPD-10A*VP* and SPD-10AV*VP* are accessible from the front of the detector, for faster and easier maintenance. You can reconfigure the instrument for semi-micro HPLC without modifying plumbing in the existing HPLC system.
Solvent Recycle Kit	The optional solvent recycle valve kit decreases isocratic HPLC mobile phase consumption by recycling clean discharge. A detector threshold setting determines whether solvent is sent to the waste container or mobile phase reservoir. This kit reduces operating costs and benefits the environment.

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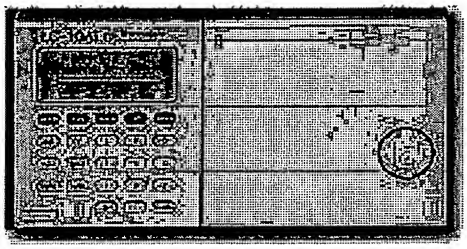

LC-10ADvp/LC-10ATvp

Solvent Delivery Units for Shimadzu VP Series HPLC System

The VP Series offers two solvent delivery units. Choose the one that's right for your HPLC application. Shimadzu pumps have built-in validation functions for GLP/GMP compliance, and offer front access to seals and plungers for fast maintenance. New, improved seal and plunger materials provide increased pump durability, and leak sensors are standard for added safety. Both pumps can be used for a wide array of applications. Set them up in isocratic, high-pressure gradient, or low-pressure gradient configurations.

The **LC-10ADvp** micro-plunger pump is unsurpassed for stable, pulse-free solvent delivery. It is perfect for low flow rate semi-micro and LC/MS applications as well as for high sensitivity analyses.

The **LC-10ATvp** serial dual plunger pump is extremely durable. It is well-suited for routine analyses and low pressure gradient operations.

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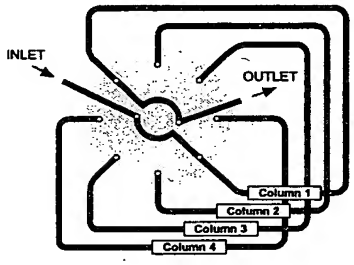
Both column ends selected – ST configuration

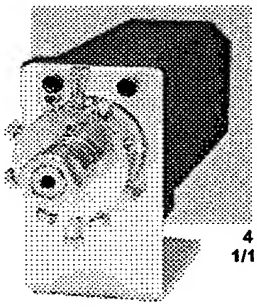
ST valves are used for multi-column, multi-sample, or multi-trap operations. This valve can be used between an injector and detector to permit manual or automated HPLC column selection. For an application suggestion, see page 161.



1/16" fittings, 0.40 mm ports (.016") *UW Type*

Standard electric actuators: 110 VAC for USA; 110/230 VAC to 24 VDC power supply for international.
Microelectric actuators: 24 VDC, with 110/230 VAC to 24 VDC power supply.

	4 Columns or Loops *Prod No*	6 Columns or Loops *Prod No*
Manual (not recommended)	CST4UW	CST6UW
With air actuator	ACST4UW	ACST6UW
With standard electric actuator	ECST4UW	ECST6UW
With microelectric actuator	EMTCST4UW	EMTCST6UW
Replacement valve	DCST4UW	DCST6UW
Replacement rotor	SSACST4UW	SSACST6UW



ST 4 position 1/16" fittings

1/16" Stainless steel loops *for UW Type valves*

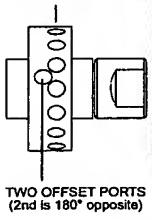
Each stainless steel loop includes two stainless nuts and two stainless ferrules. Order special fittings separately.
When a set of loops is ordered, loops will be supplied from the same lot.

Volume	*Prod No*	*Volume*	*Prod No*
10 µl	SL10CSTUW	250 µl	SL250CSTUW
15 µl	SL15CSTUW	500 µl	SL500CSTUW
20 µl	SL20CSTUW	1 ml	SL1KCSTUW
25 µl	SL25CSTUW	2 ml	SL2KCSTUW
50 µl	SL50CSTUW	5 ml	SL5KCSTUW
100 µl	SL100CSTUW	10 ml	SL10KCSTUW

FURTHER REFERENCE

ABOUT LOOPS

- Other materials available in many sizes: Electroformed Nickel, Hastelloy C, Nickel 200, PEEK, PTFE, and Titanium
- Loops > 2 ml are made from 1/8" OD tubing with brazed or welded 1/16" tube ends or reducing unions.



SPECS

5000 psi liq
75°C max

Valve body:
Nitronic 60 Stainless

Rotor:
Valcon E

Trap Cartridges

Michrom BioResources, Inc. offers a variety of trap cartridges packed with application specific HPLC column packing materials for concentration, desalting, detergent removal and protein removal from samples prior to analysis by HPLC, LC/MS, MALDI-MS, Edman sequencing and/or amino acid analysis. These cartridges can be used individually or in series to cleanup samples manually, online using a HPLC injector or automatically with a HPLC autosampler. These application specific cartridges are available in three sizes: capillary (CapTrap™), microbore (MicroTrap™) and macrobore (MacroTrap™), with capacities as shown in the table below.

Cartridge Type	Bed Volume	Sample Capacity	Sample Volume	Speed of Loading
CapTrap (0.5 X 2 mm)	0.5 ul	2 ug	0.1 - 100 ul	5 - 20 ul/min
MicroTrap (1 X 8 mm)	5.0 ul	20 ug	1.0 - 1000 ul	50 - 200 ul/min
MacroTrap (3 X 8 mm)	50 ul	200 ug	10 - 10000 ul	500 - 2000 ul/min

Click on the following links to get information on the types of Trap Cartridges

Amino Acid Traps	ISRP Traps	NID Traps	Peptide Traps
Protein Traps	SCX Peptide Traps	SDS Traps	Small Molecule Traps

Manual Sample Preparation

Samples can be cleaned up without the use of a HPLC by using a manual system as shown in Figures 1 and 2.

Figure 1. Manual CapTrap Holder Kit With Micro-Macro Holder and 10-32 Male/Male Union. For Use in Two Step Applications Such as SDS Removal From Proteins and Peptides.

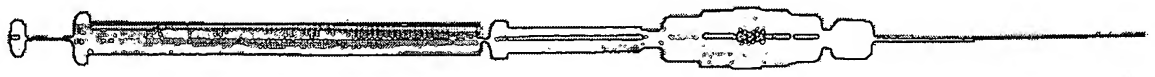


Figure 2. Manual Micro - Macro Trap Holder Kit

All Manual Holder Kits Include: Syringe, filler port, trap holder, and outlet tube for sample elution

Holder Description	Part Number	Syringe Size	Sample Elution Volume
Manual CapTrap Holder Kit	602/25029/03	*10 ul*	*For 1-5 µl sample elution*
Manual MicroTrap Holder Kit	004/25111/01	*25 ul*	*For 5-25 ul sample elution*
Manual MacroTrap Holder Kit	004/25111/02	*100 ul*	*For 20 -100 ul sample elution*

On-Line Sample Preparation

Samples can be cleaned up on-line using a manual or automated HPLC inject r with the trap cartridge built into the loop, as shown in Figures 3 and 4. Dilute samples can easily be concentrated and then washed with the starting HPLC mobile phase to remove salts and other non volatile buffers prior to analysis by HPLC, LC/MS or FIA/MS. Michrom's trap cartridges can also be used with the MAGIC HPLC autosampler (and other autosamplers with sample preparation capabilities) to automate concentration, desalting and detergent or protein removal from a variety of biological and pharmaceutical samples.

Loop/Holder Includes: Trap holder and Two Connecting Tubes with Fittings

Holder Description	Part Number
Valco CapTrap Loop/Holder	602/25029/00
Rheodyne CapTrap Loop/Holder	602/25029/01
Valco MicroTrap Loop/Holder	004/25110/00
Rheodyne MicroTrap Loop/Holder	004/25111/00
Valco MacroTrap Loop/Holder	004/25110/00
Rheodyne MacroTrap Loop/Holder	004/25111/00

Capillary: 50u ID PEEKSIL Tubing. Total Holder, Trap & Tubing Volume is <1 ul
Micro: 0.007" ID PEEK Tubing. Total Holder, Trap & Tubing Volume is <10 ul
Macro: 0.007" ID PEEK Tubing. Total Holder, Trap & Tubing Volume is <60 ul

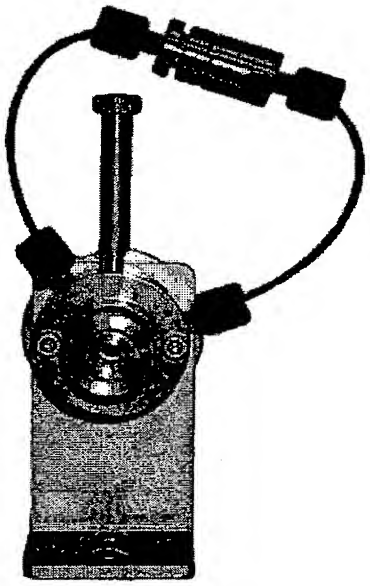


Figure 3. Valco Manual HPLC Injector With CapTrap Loop/Holder and Syringe Filler Port.

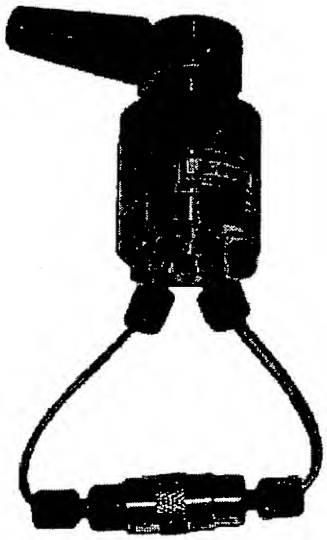
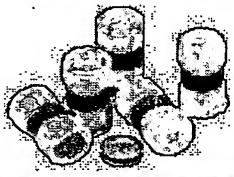
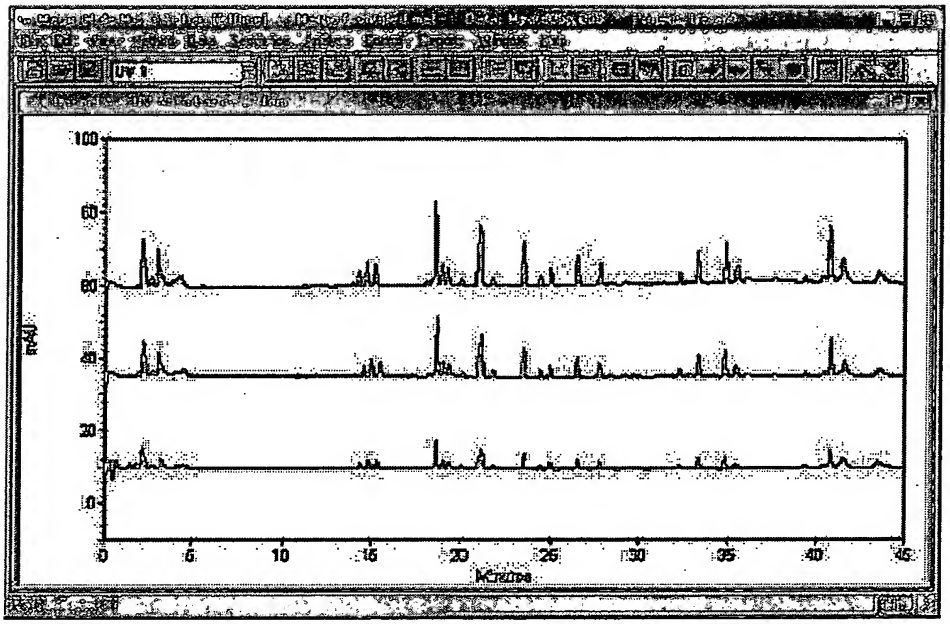


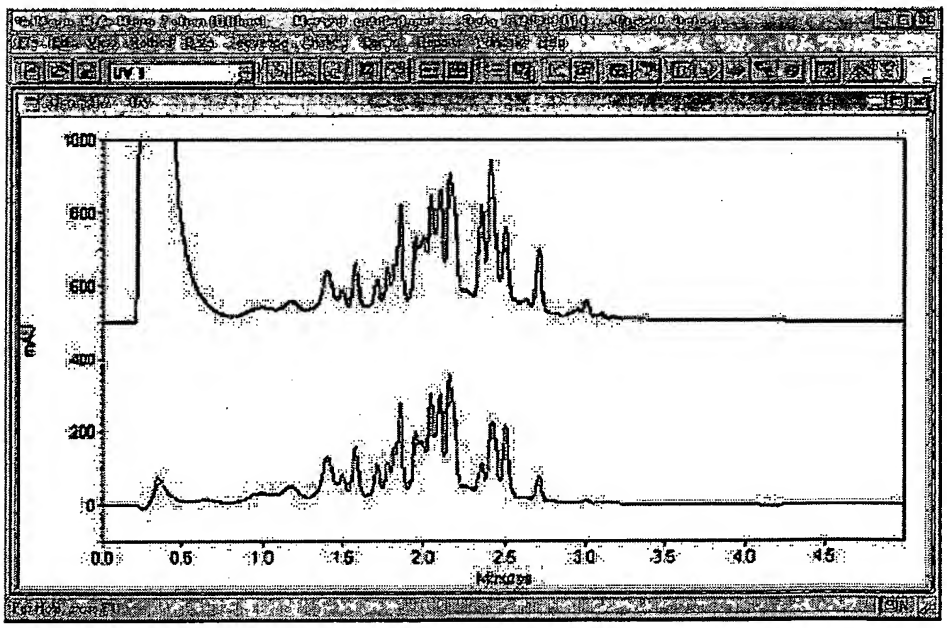
Figure 4. Rheodyne Manual HPLC Injector With Micro - Macro Trap Loop/Holder.

Peptide Concentration & Desalting Traps





On-line concentration of a dilute protein digest from a 2D gel (1 pmol/ml) on a peptide microtrap for trace LC/MS analysis



Automated desalting of recombinant protein digest on a peptide macrotrap for fast LC/MS quality control

The Michrom Peptide Trap Cartridges contain a medium pore, large particle, polymeric reversed-phase HPLC column packing that is designed to bind protein digests, peptides, small polyneucleotides and other small biological molecules (0.5 – 50 kD). The peptide trap is used to concentrate samples and remove salts, buffers and other small, polar molecules prior to analysis or characterization using HPLC, LC/MS, MALDI TOF-MS, Edman Sequencing or other techniques. The peptide trap can also be used in series with a SDS removal trap for samples that require SDS detergent removal as well as concentration and/or desalting. Michrom's peptide traps can be used to concentrate samples up to 100 fold and can effectively remove salts and nonvolatile buffers to greater than 99.9%. The polymeric packing material allows binding of moderately hydrophilic compounds and most small component biological samples in as little as 1% organic solvent, yielding excellent recovery (>90% at levels above 10 femtomoles). The Michrom Peptide Trap Cartridges can be cleaned with strong acid, strong base and/or organic solvents to eliminate carry-over and allow the trap to be used for hundreds to thousands of samples. For large hydrophobic peptides (ie CNBr digests) the protein trap may be more appropriate, while for very small hydrophilic peptides (ie phosphopeptides) the small molecule trap may be more appropriate.

Description	Band Color	Capacity	Part Number
Peptide MacroTrap (Each)	Green	200 ug	004/25108/52
Peptide MacroTrap (6 Pack)	Green	200 ug	004/25109/52
Peptide MicroTrap (Each)	Green	20 ug	004/25108/02
Peptide MicroTrap (6 Pack)	Green	20 ug	004/25109/02
Peptide CapTrap (Each)	Green	2 ug	004/25108/32
Peptide CapTrap (6 Pack)	Green	2 ug	004/25109/32

Anal. Chem. **1997**, *69*, 1518–1524

Comprehensive On-Line LC/LC/MS of Proteins

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This is a description of a comprehensive two-dimensional liquid chromatography (LC) system for the separation of protein mixtures. This system uses cation-exchange chromatography followed by reversed-phase chromatography (RPLC). The two LC systems are coupled by an eight-port valve equipped with two storage loops and under computer control. The RPLC effluent is sampled by both a UV detector and an electrospray mass spectrometer. In this way, complex mixtures of large biomolecules can be rapidly separated, desalted, and analyzed for molecular weight in less than 2 h. The system's utility is demonstrated with a mixture of standards and an *Escherichia coli* cell lysate.

Comprehensive LC/LC systems were invented to simplify the separation of proteins which overwhelm one-dimensional LC systems[1,2] or even hyphenated techniques such as CE–MS or LC–MS. These comprehensive techniques differ from ordinary heart cutting two-dimensional (2D) systems[3–9] in that they subject the entire sample to 2D separation. The two dimensions should ideally be orthogonal,[10] and any separation accomplished by the first dimension again should ideally be retained upon transfer to the second dimension. The coupled column or heart cutting systems usually work by trapping a few analytes of interest and then separating those by reversed-phase LC. All other sample components are diverted to waste. In a comprehensive system, all analytes are acted upon equally by both dimensions, without the diversion of only a few species to the second dimension. This LC-based system leaves the analytes in solution as opposed to being bound in a matrix, as does two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis[11,12] (2D-PAGE).

Until this time, the only report of coupling a comprehensive 2D separation to mass spectrometry made use of reversed-phase chromatography followed by capillary electrophoresis for the analysis of peptides.[13] Unfortunately, the physical constraints of that system make it impossible to collect analytes after they have been separated and identified. Other reports of comprehensive 2D systems used either laser-induced fluorescence for the detection of peptides[14,15] or UV exclusively for the detection of proteins.[16–19]

The system described here for the separation of protein mixtures comes close to Giddings's criteria[20] for an ideal 2D system, as it makes use of cation-exchange chromatography followed by reversed-phase chromatography, two orthogonal modes. Furthermore, since the RPLC column samples the first dimension two or three times per peak, this system tends not to recombine components already separated by the cation column. By making use of liquid chromatography, this system allows fractions to be readily collected following separation. Finally, this is the first report of comprehensive LC/LC utilizing mass spectrometry for detection, which yields on-line molecular weight information. This adds, in essence, a third dimension to this 2D system, because the mass spectrometer can identify the presence of coeluting peaks when they are not resolved by chromatography.

EXPERIMENTAL SECTION

Overview. The basic layout of the system draws on the design of Bushey and Jorgenson.[16] Figure 1 is a schematic of the new system. A pump delivers mobile phase to the injector and onto a cation-exchange column. This column's outlet is attached to a two-position, eight-port valve. As one loop fills with effluent from the ion-exchange column, the other loop is being pumped out by a second HPLC pump and through the RPLC column. This column's effluent flows through a UV detector and is split, and 10% flows to the mass spectrometer.

Liquid Chromatography. The cation-exchange chromatography begins with a dual-syringe pump (ABI 140B, Perkin Elmer,

(1) Davis, J. M.; Giddings, J. C. *Anal. Chem.* **1985**, *57*, 2168–2177.
(2) Davis, J. M.; Giddings, J. C. *Anal. Chem.* **1985**, *57*, 2178–2182.
(3) Kassel, D. B.; Consler, T. G.; Shalaby, M.; Sekhri, P.; Gordon, N.; Nadler, T. In *Techniques in Protein Chemistry VI*; Crabb, J. W., Ed.; Academic: San Diego, CA, 1995; pp 39–46.
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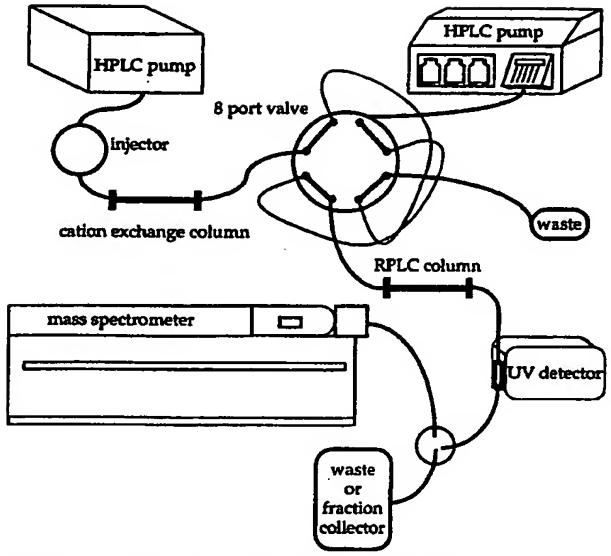

Figure 1. Schematic of LC/LC/MS instrumentation.

Norwalk, CT) delivering mobile phase to a 2.5 μL injector (Rheodyne, Cotati, CA). The column is packed in-house with Bakerbond Wide-Pore Carboxy-Sulfon 5 μm material (J.T. Baker, Phillipsburg, NJ), and its dimensions are 750 μm i.d. × 12.5 cm length. The mobile phase gradient consists of A = 50 mM sodium formate (Sigma, St. Louis, MO), 3 M urea, and 10% acetonitrile (J.T. Baker) at pH = 4, B = 1000 mM ammonium formate, 3 M urea, and 10% acetonitrile at pH = 6. A typical gradient runs from 20% B to 100% B in 2 h at 10 μL/min.

The chromatography system is controlled from a Macintosh Quadra 900 (Apple Computer, Cupertino, CA) running LabView software (National Instruments, Austin, TX) and using an NB-MIO-16XL board (National Instruments) for valve switching, triggering the mass spectrometer, and collecting UV data. The valve is an eight-port, two-position valve (EC8W, Valco Instruments, Houston, TX). Loop volume is determined by multiplying the flow rate of the ion-exchange column by the sum of the run and reequilibration times of the RPLC column.

The reversed-phase column is packed in-house with POROS R2/H material (PerSeptive Biosystems, Framingham, MA), and its dimensions are 500 μm i.d. × 10 cm length. It is supplied with mobile phase from an HP 1090 (Hewlett Packard, Palo Alto, CA), which is flow-split from 5 mL/min to 50 μL/min. Mobile phase A is water with 0.1% trifluoroacetic acid, and B is acetonitrile with 0.1% TFA. A typical gradient runs from 22% B to 100% B in 1.25 min, followed by a 0.25 min reequilibration step at initial conditions. All connections are through 62 μm i.d. × $^1/_{16}$ in. PEEK until the outlet of the RPLC column. From this outlet to the splitter, a 150 μm i.d. × 360 μm o.d. fused silica capillary is used for on-capillary UV detection (Model 200, Linear Instruments,

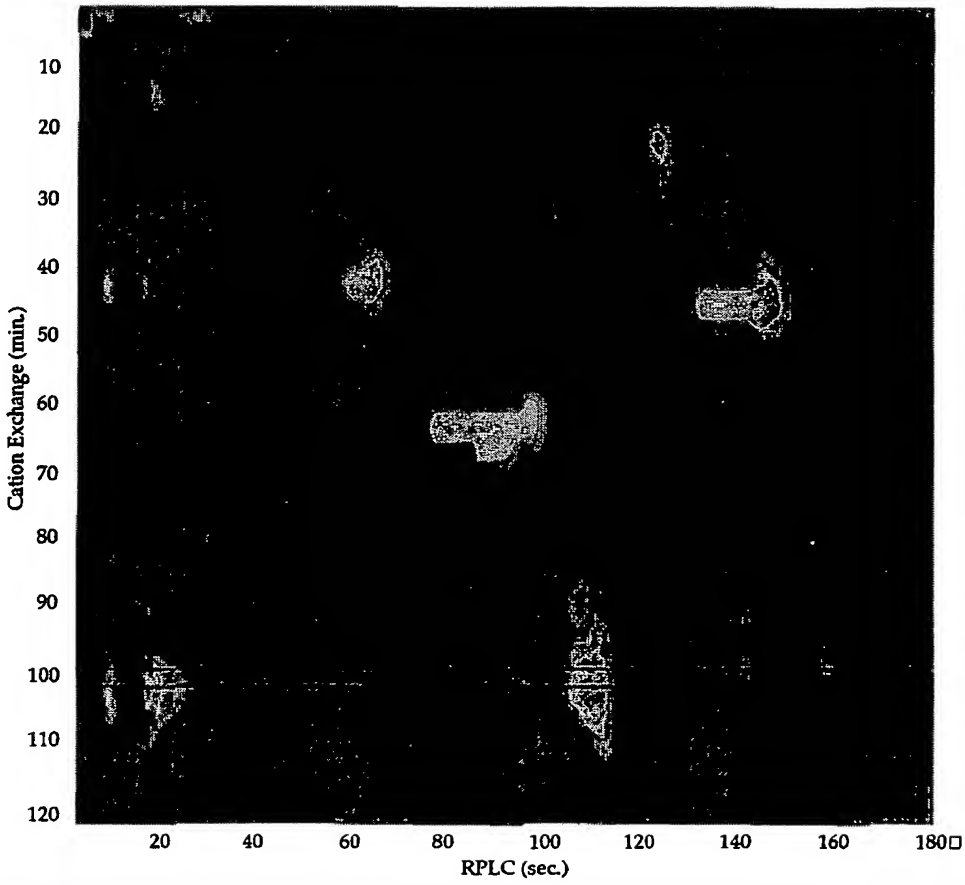

Figure 2. 2D chromatogram of protein standards: peak A, α-Lac; B, RNA; C, CAH; D, β-Lac A; E, β-Lac B; F, HH Cyt *c*; G, BH Cyt *c*; H, Lys; I, α-Hb; J, β-Hb.

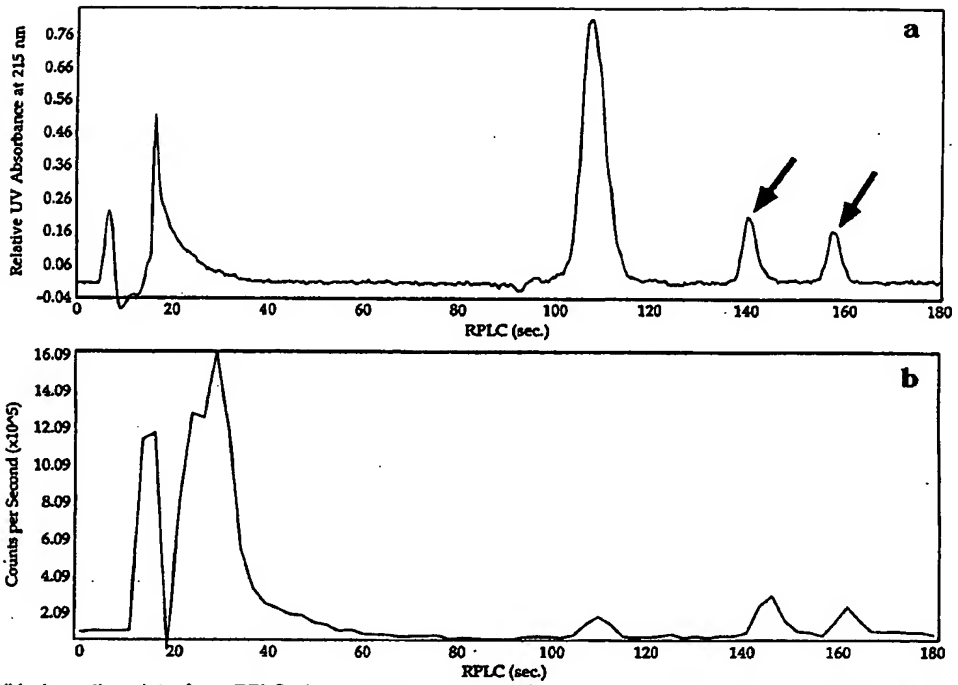

Figure 3. (a) UV absorption data from RPLC chromatogram extracted from 100 min of Figure 2. (b) Corresponding total ion current chromatogram.

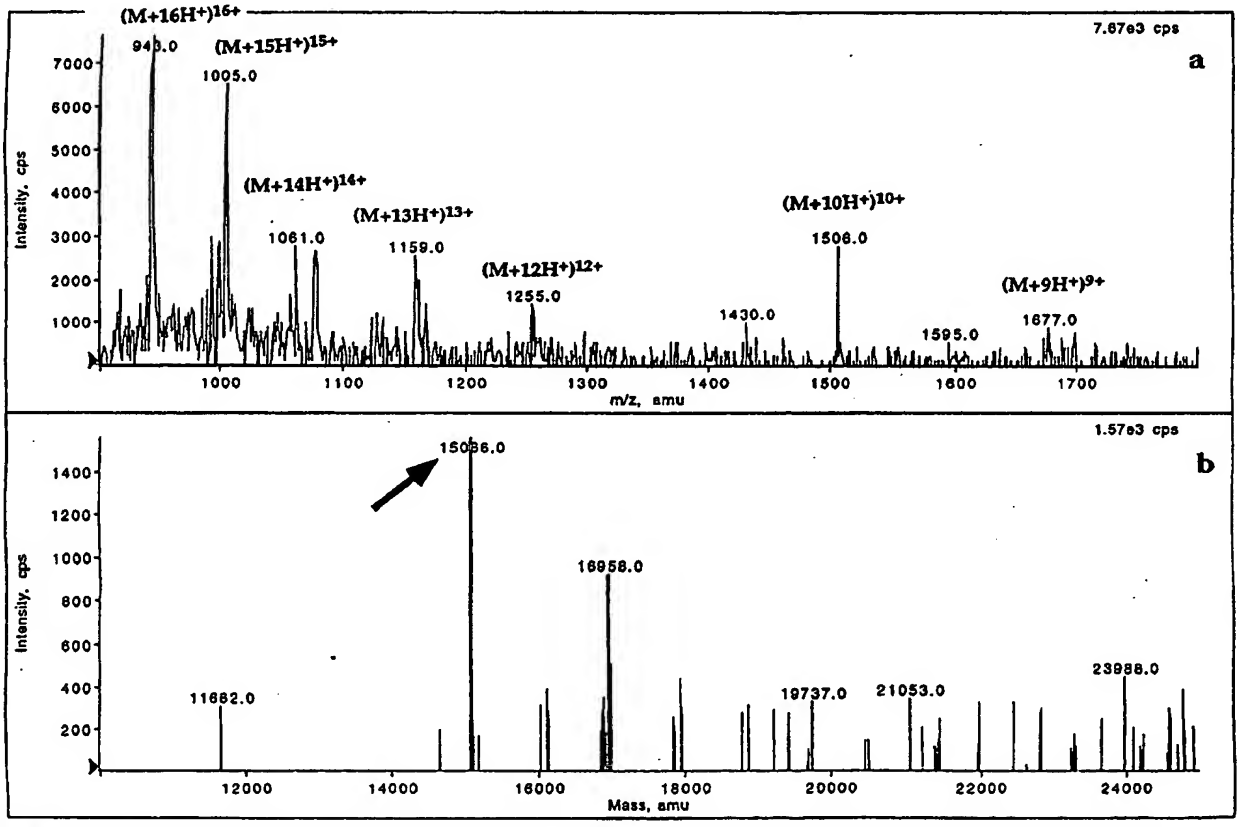

Figure 4. (a) Mass spectrum from peak at 142 s of Figure 3. (b) Corresponding Hypermass reconstruction of charge envelope.

Reno, NV). Following the splitter, a 29 μm i.d. × 150 μm o.d. capillary supplies the electrospray interface.

Mass Spectrometry. The mass spectrometer is a Sciex API 100 (Perkin Elmer) and is controlled by a Macintosh 8100 using

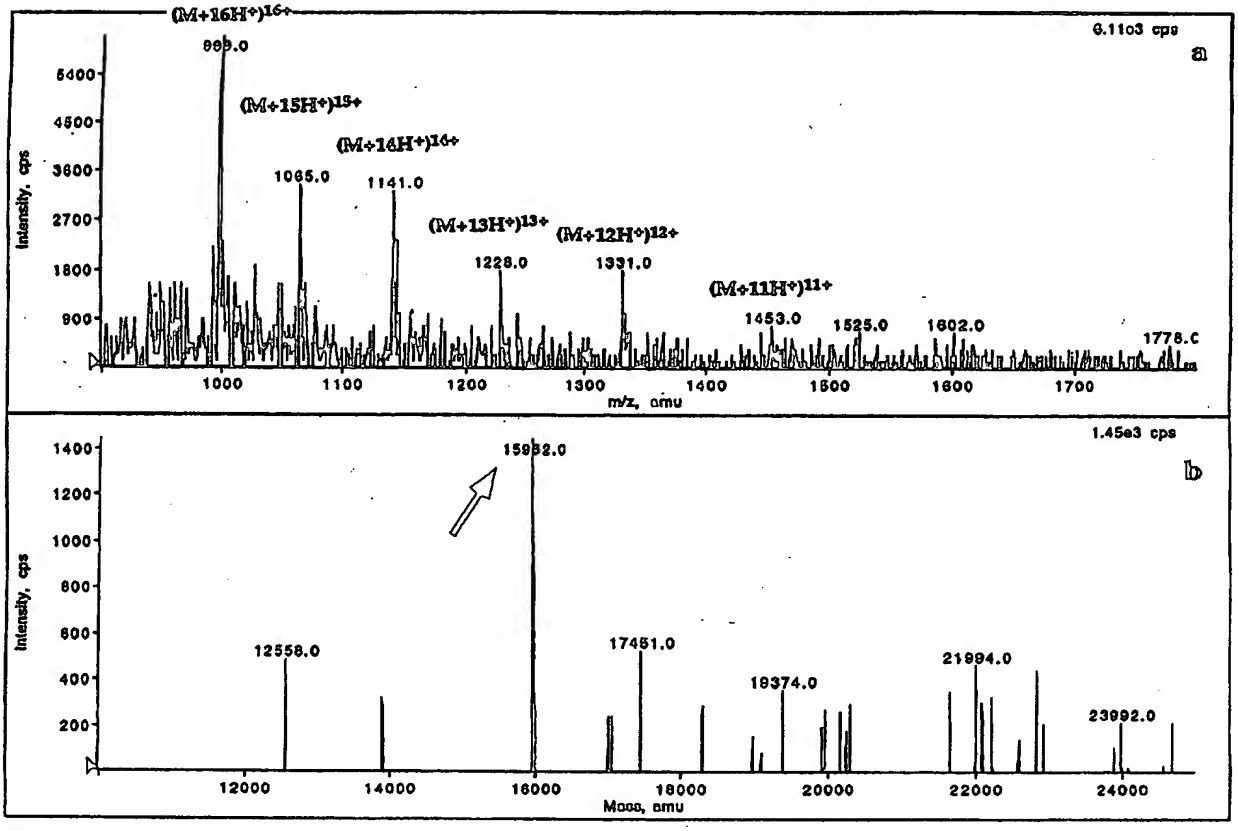

Figure 5. (a) Mass spectrum from peak at 162 s of Figure 3. (b) Corresponding Hypermass reconstruction of charge envelope.

either the supplied Sample Control or LC2Tune software. It scans from m/z 1000 to 2000 in 0.1 steps with a dwell of 0.2 ms, for a total single scan time of 2 s. If the LC2Tune program is used, the entire 2D data set is recorded as a single experiment whose duration is that of the cation-exchange run time plus one RPLC run. While this collects all data, including the salt peak and reequilibration time, it stores this data in volatile random access memory (RAM). This limits the number of data points that can be collected to the computer's RAM and is susceptible to loss in the event of a power interruption or surge. Another way to collect the mass spectral data is to use the Sample Control program, which receives a start signal at the beginning of each RPLC run and collects until 12 s from the end of that run, when it bundles the data, writes it to disk, and waits for the start of the next RPLC run. With this method, only the current RPLC run is stored in volatile memory, and 2D experiments are limited only to the size of the hard disk drive.

Data Workup. Data from the UV detector are background subtracted with a LabView program written in-house and displayed using Transform 3.0 (Fortner Research, Sterling, VA). The mass spectrometer data are analyzed in Bio-Multiview (ver. 1.0, Perkin-Elmer), which calculates the molecular weight of a chromatographic peak from its charge envelope.

Sample Preparation. Protein standards are purchased from Sigma, dissolved in IEC mobile phase A, and filtered through 0.45 μm filters (UFC3 OHV 25, Millipore, Bedford, MA). The standards used are α-lactalbumin from bovine milk (α-Lac), ribonuclease A(RNA) from bovine pancreas, β-lactoglobulins A and B from bovine milk (β-Lac A, β-Lac B), carbonic anhydrase from bovine erythrocytes (CAH), cytochrome *c* from horse heart and bovine heart (HH Cyt *c*, BH Cyt *c*), bovine hemoglobin (α-Hb, β-Hb), and lysozyme from chicken egg white (Lys). Each protein's concentration in the standard mixture is 1 mg/mL, except the β-lactoglobulins, which are 0.5 mg/mL each. The *Escherichia coli* sample comes as intact cells in the fermentation broth. The cells are centrifuged to the bottom of the tube at 6000*g* for 1 h. The broth is removed, and the cells are resuspended in an equivalent volume of IEC mobile phase A for a total protein concentration of ~7.5 mg/mL, determined by amino acid analysis. The cells are sonicated in a cold water bath for 30 min and recentrifuged. The supernatant is withdrawn and filtered through a 0.45 μm filter.

RESULTS

Figure 2 shows the separation of 10 protein standards as detected by UV absorption at 215 nm. A component's coordinates are obtained by observing its retention time on the cation-exchange column from the *y*-axis and its RPLC retention time from the *x*-axis. In this case, one 120 min cation-exchange run is sampled by 48 individual 150 s duration RPLC runs. The streak down the *y*-axis between 5 and 20 s of the RPLC runs results from the salt and urea in the first dimension. At 40 and 100 min (IEC run time), the intensity of the salt and urea peak, components which are unretained on the RPLC column, rises out of the background of the 2D plot. At these times, the simultaneous injection of a large quantity of salt, urea, and protein saturates

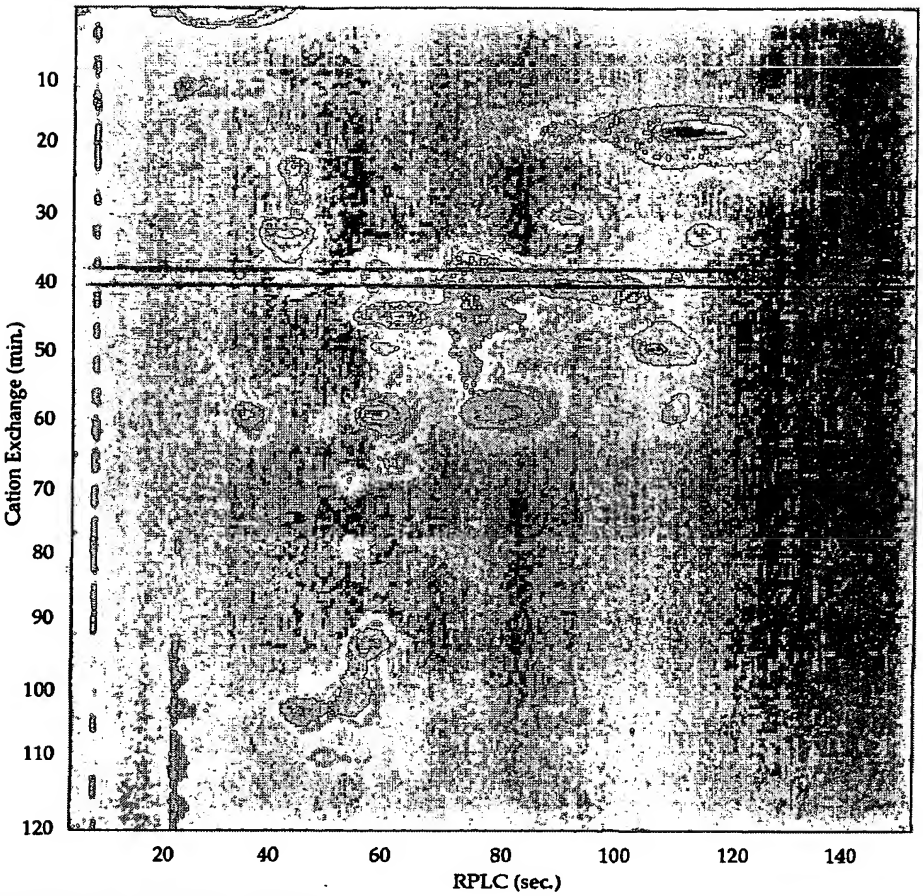

Figure 6. 2D chromatogram of *Escherichia coli* lysate.

the 750 μm i.d. RPLC column, which causes the unretained peak to tail. Since the data for the 2D plot are background subtracted using an RPLC run with only a salt and urea injection, the tailed peak creates the artifacts observed in the RPLC runs beginning at 40 and 100 min. These are the only places such an event occurs in the 2D plot because the total salt, urea, and protein loads during other RPLC runs are not sufficient to saturate the RPLC column.

The shapes of peaks in the 2D plot are examined in closer detail by extracting the individual RPLC run(s) in which they appear. For example, the individual RPLC run at 100 min of the IEC run is shown in Figure 3, where both the UV absorption and total ion intensity data are plotted. Peaks are observed at 112, 142, and 162 s in the UV and slightly later in the ion current data because of the void volume between the detectors. The peaks in the ion current appear broader than those in the UV because of the lower data acquisition rate of the mass spectrometer (0.5 vs 4 Hz). The mass spectra of the two later-eluting peaks make up Figures 4a and 5a. The Hypermass reconstruction of these peaks, shown below each spectrum in Figures 4b and 5b, identifies them as the two chains of hemoglobin. The last-eluting peak results from an injection onto the front of the system of 16 pmol of intact hemoglobin or 32 pmol of the β-chain. The entire peak flows through the UV detector, but the mass spectrometer, because it follows the 10:1 split, is presented with 3.2 pmol.

Reexamining Figure 2 shows that most peaks eluting off of the IEC column are 6 min wide. The time between the dead volume and the last-eluting peak is 100 min. This equals a peak capacity of 16 in the first dimension. Returning to Figure 3a, the peak width is about 5 s; dividing the available elution time of 160 s (excluding the dead time) by this, the peak capacity is 32 in the second dimension. Multiplying 16 by 32 produces a chromatographic peak capacity of 512. The mass spectrometer also has inherent peak capacity, conservatively taken to be 5, because it can identify at least this number of components while scanning a 1000 m/z range. This makes the entire LC/LC/MS system's peak capacity greater than 2500.

The system is tested with a sample of pharmaceutical interest, a bacterial cell lysate often used to overexpress proteins for work in screening and structural studies. In this case, the chromatographic conditions are very similar to those used in the run of standards, only now the ion-exchange gradient begins at 14% instead of 20%. The UV absorbance data from the run of the *E. coli* cell lysate are shown in Figure 6. The extracted UV and ion current traces at 37.5 min make up Figure 7. The peak at 110 s in this RPLC run has the mass spectrum shown in Figure 8a, with its reconstructed molecular weight shown as 40 702 in Figure 8b. A search of the Swiss-Prot database shows 38 proteins from *E. coli* within 2% of this molecular weight but only two proteins within 0.2%, GCPE protein (P27433) and hydrogenase-1 small-chain precursor (P19928).

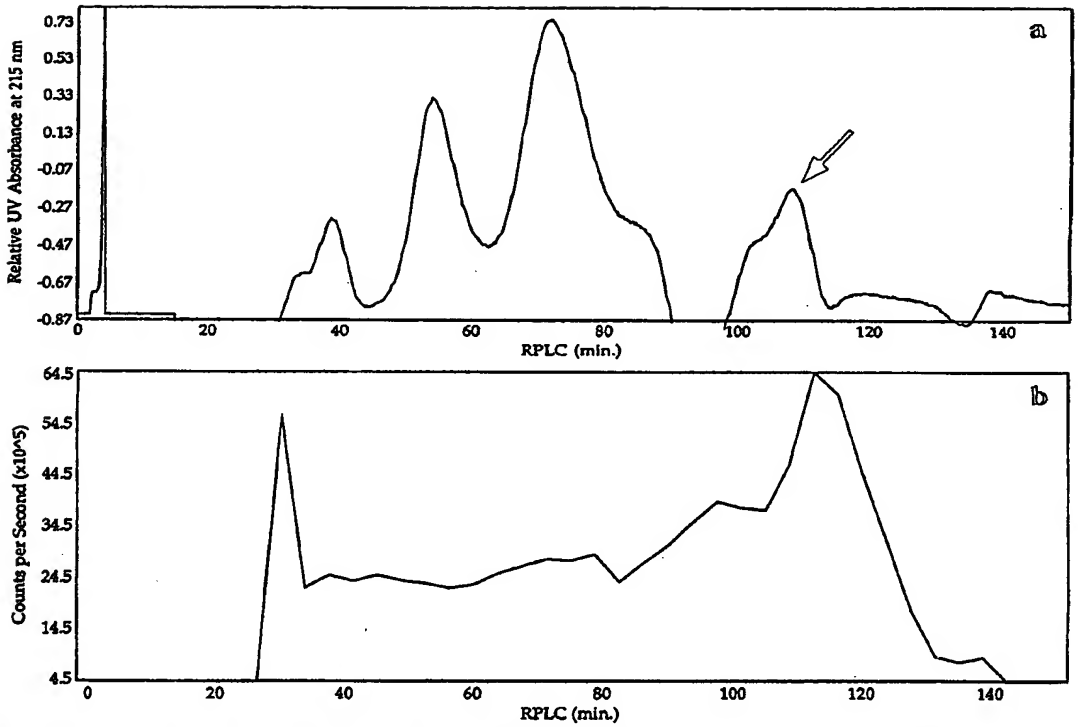

Figure 7. (a) UV absorption data from RPLC chromatogram extracted from 37.5 min of Figure 6. (b) Corresponding total ion current chromatogram.

DISCUSSION

A comprehensive 2D system without mass spectrometry can identify sample components on the basis of retention time. Of course, this is even more rigorous than a one-dimensional system because of the unlikely possibility that two components will have the same retention time in two orthogonal modes of separation. However, for complex samples that are uncharacterized, the addition of mass spectrometry as a detector to the system makes the system very capable of quickly screening for the major components, as can be seen in the run of *E. coli* lysate (Figure 7). At the end of the run, an analyte's retention times in two modes of chromatography, as well as its molecular weight within an accuracy of 0.2%, are known.

Compared to 2D polyacrylamide gel electrophoresis, this LC/LC systems pales with regard to peak capacity (>5000) and sensitivity (femtomole). However, to get a more precise molecular weight than that provided by the sodium dodecyl sulfate dimension, or to obtain the N-terminal sequence, a spot must be excised from the gel. This sample needs to be separated from the matrix, the SDS, and any stain used to develop the gel. These steps require a fair amount of operator effort and can introduce sample contamination and loss. The LC/LC/MS system described here, like the LC techniques it incorporates, is clearly faster and less prone to sample loss than 2D-PAGE. Additionally, the proteins can be recovered intact because of the 10:1 flow-split before the mass spectrometer. This allows for off-line proteolysis, Edman sequencing, or amino acid analysis.

Ion-exchange and reversed-phase modes of chromatography are used because they offer high-resolution separations of proteins. They are placed in such an order for two reasons. First, because of the necessity to run the second dimension quickly in order to minimize the recombination of components eluting off the first dimension, a stationary phase with a high optimum flow rate must be used. Only such reversed-phase materials are readily available and offer a large body of knowledge about their performance at high flow rates. Second, in order to couple to the mass spectrometer, the mobile phase of the second dimension must be amenable to electrospray ionization, again suggesting RPLC. These chromatographic modes are implemented on a microcolumn scale to improve efficiency as well as to reduce sample dilution for the electrospray ionization mass spectrometry, a concentration-sensitive technique.

Another way to look at the utility of this system is to recognize its ability to desalt the proteins on-line. By using a system such as this, established ion-exchange methods of protein separations can now be used as the basis for an LC–MS system, instead of having to discard those methods in favor of reversed-phase chromatography. A simple step gradient could be used in the second dimension if on-line desalting was the only goal. Since the cation-exchange chromatography is currently run at or below the optimum linear velocity in order to "broaden" the peaks, a step gradient would allow the first dimension to be run at higher linear velocities, reducing the time required to complete an experiment.

The UV trace and the ion current data both respond well to all 10 components in the standard mixture, but even the two weakest, α- and β-Hb, still show acceptable signals. Occasionally, a protein signal will be particularly broad and thus dilute. This often means that the ion current trace shows very little evidence of a peak. By having the UV detector in series, such a dilute peak can often be located from the absorbance data, allowing the

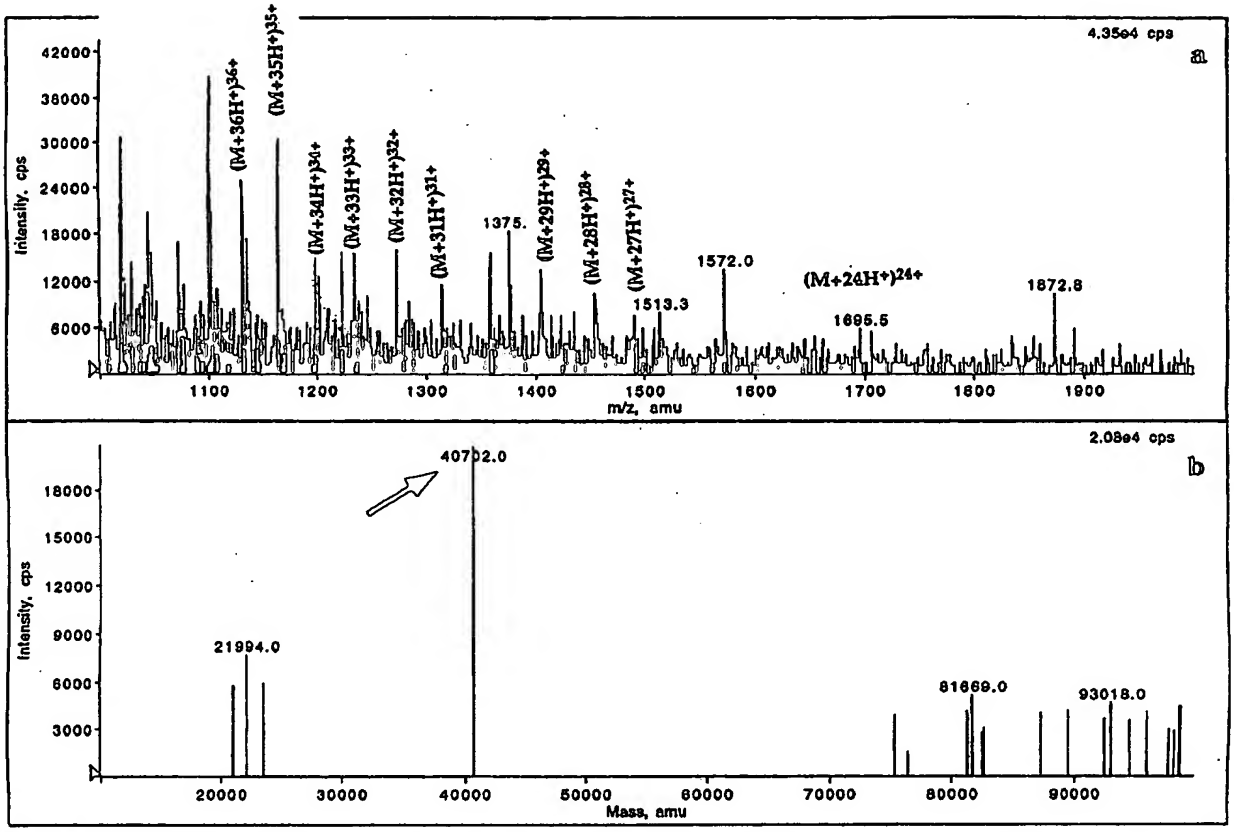

Figure 8. (a) Mass spectrum from peak at 110 s of Figure 7. (b) Corresponding Hypermass reconstruction of charge enve lope.

operator to target a certain region of mass spectral data in order to elucidate a charge envelope. Should the component not yield a usable mass spectrum, there exists another option. With the system described in this report, in lieu of one utilizing capillary electrophoresis for the second dimension, it is possible to collect fractions for off-line analysis.[21] All that is necessary is a fraction collector after the 10:1 flow split and before the mass spectrometer.

Numerous possibilities exist to improve this system. Perhaps the easiest is to increase the column length in the first dimension in order to increase efficiency. Unfortunately, this results in an increase in analysis time. A more efficient second dimension using a high flow rate stationary phase based on 5 instead of 10 μm particles would also be desirable. Not only would this increase the peak capacity and resolving power of the system, but it would also enhance the sensitivity of the system by making the peaks sharper and less dilute. Small increases in either dimension result in large overall gains in peak capacity, again because of the multiplicative effect of combining two systems in a comprehensive manner.

CONCLUSIONS

A comprehensive LC/LC system has been coupled to electrospray mass spectrometry in order to determine the molecular weights of proteins on-line. An entire mixture is separated in two orthogonal dimensions, without any prior knowledge of the individual components, e.g., p*I*, molecular weight, hydrophobicity, affinity. By coupling electrospray ionization mass spectrometry to an efficient chromatography system, a total peak capacity of over 2500 is obtained. The mass spectrometer can be presented with as little as 3.2 pmol of analyte and still obtain an accurate molecular weight. The use of reversed-phase chromatography allows the concentrated salt and urea ion-exchange mobile phase to be coupled directly to the mass spectrometer.

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